

## Increased bioavailability of cyclic guanylate monophosphate prevents retinal ganglion cell degeneration



Lauren K. Wareham<sup>a,b,1</sup>, Ana C. Dordea<sup>a</sup>, Grigorij Schleifer<sup>a</sup>, Vincent Yao<sup>a,b,1</sup>, Annabelle Batten<sup>a</sup>, Fei Fei<sup>b</sup>, Joseph Mertz<sup>c</sup>, Meredith Gregory-Ksander<sup>g</sup>, Louis R. Pasquale<sup>d</sup>, Emmanuel S. Buys<sup>a</sup>, Rebecca M. Sappington<sup>b,e,f,\*</sup>

<sup>a</sup> Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital Research Institute, Harvard Medical School, Boston, MA, USA

<sup>b</sup> Vanderbilt Eye Institute, Vanderbilt University Medical Center, Nashville, TN 37232, United States

<sup>c</sup> Department of Ophthalmology, Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

<sup>d</sup> Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA

<sup>e</sup> Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232, United States

<sup>f</sup> Department of Ophthalmology and Visual Sciences, Vanderbilt University School of Medicine, Nashville, TN 37232, United States

<sup>g</sup> Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, MA 02114, United States

### ARTICLE INFO

#### Keywords:

cGMP  
Retinal ganglion cell  
Glaucoma  
Nitric oxide  
PDE5

### ABSTRACT

The nitric oxide – guanylyl cyclase-1 – cyclic guanylate monophosphate (NO-GC-1-cGMP) pathway has emerged as a potential pathogenic mechanism for glaucoma, a common intraocular pressure (IOP)-related optic neuropathy characterized by the degeneration of retinal ganglion cells (RGCs) and their axons in the optic nerve. NO activates GC-1 to increase cGMP levels, which are lowered by cGMP-specific phosphodiesterase (PDE) activity. This pathway appears to play a role in both the regulation of IOP, where reduced cGMP levels in mice leads to elevated IOP and subsequent RGC degeneration. Here, we investigated whether potentiation of cGMP signaling could protect RGCs from glaucomatous degeneration. We administered the PDE5 inhibitor tadalafil orally (10 mg/kg/day) in murine models of two forms of glaucoma – primary open angle glaucoma (POAG; GC-1<sup>-/-</sup> mice) and primary angle-closure glaucoma (PACG; Microbead Occlusion Model) – and measured RGC viability at both the soma and axon level. To determine the direct effect of increased cGMP on RGCs *in vitro*, we treated axotomized whole retina and primary RGC cultures with the cGMP analogue 8-Br-cGMP. Tadalafil treatment increased plasma cGMP levels in both models, but did not alter IOP or mean arterial pressure. Nonetheless, tadalafil treatment prevented degeneration of RGC soma and axons in both disease models. Treatment of whole, axotomized retina and primary RGC cultures with 8-Br-cGMP markedly attenuated both necrotic and apoptotic cell death pathways in RGCs. Our findings suggest that enhancement of the NO-GC-1-cGMP pathway protects the RGC body and axon in murine models of POAG and PACG, and that enhanced signaling through this pathway may serve as a novel glaucoma treatment, acting independently of IOP.

### 1. Introduction

Glaucoma, a common optic neuropathy, is the leading cause of irreversible blindness worldwide. By the year 2040, an estimated 111 million people will have glaucoma, many of which will be bilaterally blind (Quigley & Broman, 2006; Tham et al., 2014). Glaucoma is characterized by degeneration of retinal ganglion cells (RGCs), whose axons form the optic nerve (Shaikh et al., 2014). There are two major forms of the disease – primary angle closure glaucoma (PACG) and primary open

angle glaucoma (POAG), with the latter being most prevalent (Friedman et al., 2004). Several risk factors for glaucoma have been identified, however the only risk factor currently amenable to treatment is the level of intraocular pressure (IOP). Animal models of glaucoma illustrate a strong correlation between cumulative IOP exposure and optic neuropathy (Mabuchi et al., 2003; Yucl et al., 2003). Large clinical trials indicate that significant and sustained IOP lowering by pharmacological, laser or surgical intervention slows vision loss in glaucoma patients (Heijl et al., 2002; Garway-Heath et al., 2015).

\* Corresponding author at: Vanderbilt Eye Institute, Vanderbilt University Medical Center, Nashville, TN 37232, United States.

E-mail address: [rebecca.m.sappington@vanderbilt.edu](mailto:rebecca.m.sappington@vanderbilt.edu) (R.M. Sappington).

<sup>1</sup> Current affiliation.

<https://doi.org/10.1016/j.nbd.2018.09.002>

Received 3 July 2018; Received in revised form 9 August 2018; Accepted 3 September 2018

Available online 10 September 2018

0969-9961/ © 2018 Elsevier Inc. All rights reserved.

However, human studies demonstrate that substantial IOP lowering does not completely halt disease progression.(Heijl et al., 2002; Am J. Ophthalmol., 1998) Furthermore lowering IOP in patients with ocular hypertension slows, but does not completely prevent the onset of glaucomatous disease.(Kass et al., 2002) These findings underscore the need to find strategies that directly protect the RGCs in clinical glaucoma management.

Multiple genetic epidemiology studies implicate impaired nitric oxide – guanylate cyclase-1 – cyclic guanylate monophosphate (NO-GC1–cGMP) signaling as a possible pathogenic mechanism in POAG(Kang et al., 2010; Buys et al., 2013; Emam et al., 2014; Magalhaes da Silva et al., 2012) and PACG.(Ayub et al., 2010; Awadalla et al., 2013; Rong et al., 2016) NO activates GC-1 to increase levels of cGMP and cGMP levels are lowered by degradation via cGMP-specific phosphodiesterases (PDE). Among women the relation between *NOS3* polymorphisms and POAG is significantly modified by age at menarche, parity,(Kang et al., 2011) and postmenopausal hormone use.(Kang et al., 2010) Similarly, variants in the promoter region of *NOS3* were identified in 20% of familial POAG patients(Tunny et al., 1998) and the *NOS3* variant *rs2070744* is significantly associated with a subset of normal tension glaucoma patients with optic nerve head hemorrhage.(Jeoung et al., 2017) Furthermore, variants in the genomic region that encompasses *GUCY1A3* and *GUCY1B3*, the genes encoding the  $\alpha 1$  and  $\beta 1$  subunit of GC-1, are associated with female POAG cases that exhibit early paracentral vision loss in the Glaucoma Genes and Environment (GLAUGEN) study.(Buys et al., 2013) Likewise, genetic variants in upstream components of the NO-GC-1-cGMP pathway are also associated with open-angle glaucoma(Ozel et al., 2014; Thorleifsson et al., 2010; Wiggs et al., 2011; Nunes et al., 2018; Khawaja et al., 2018); specifically loci between *CAV1* and *CAV2*, the genes encoding for Caveolin 1/2 which control NO production by *NOS3*(Mineo & Shaul, 2012) are associated with POAG. Physiological studies suggest that NO and cGMP regulate aqueous humor (AqH) outflow, and optic nerve head hemodynamics in pre-clinical models(Buys et al., 2013; Ayub et al., 2010; Awadalla et al., 2013; Rong et al., 2016; Kang et al., 2011; Lei et al., 2015; Chang et al., 2015) and have clinical relevance for glaucoma treatment.(Ellis et al., 2009; Khoobehi et al., 2011; Kotikoski et al., 2003; Krauss et al., 2011; Weinreb et al., 2015; Weinreb et al., 2016; Kawase et al., 2016) Collectively these studies highlight a role for the NO-GC-1-cGMP pathway in POAG and PACG pathogenesis, representing important steps toward understanding the contribution of the NO-GC-1-cGMP pathway in the etiology of glaucoma.

We recently implicated a role for GC-1 in the regulation of IOP in glaucoma.(Buys et al., 2013; Muenster et al., 2017) Our findings indicated that female mice lacking the alpha-1 subunit of GC-1 (formerly soluble guanylate cyclase or sGC), develop age-related glaucoma mimicking human POAG.(Buys et al., 2013) GC-1 disruption leads to age-related elevated IOP and RGC degeneration. Here, we investigated whether preventing breakdown of cGMP by phosphodiesterase type 5 (PDE5) could, conversely, protect RGCs from glaucomatous degeneration. We found that the PDE5 inhibitor, tadalafil, increases serum cGMP levels and impedes RGC loss in murine models of both POAG (GC-1<sup>-/-</sup> mice) and PACG (Microbead Occlusion Model; MOM) without altering IOP. Additionally, we determined that cGMP directly impedes axotomy-induced necrosis and apoptosis in whole retina as well as in primary RGCs. Our findings suggest that increased bioavailability of cGMP prevents IOP-related RGC degeneration in an *IOP-independent* manner. We provide evidence for IOP-independent neuroprotection in both POAG and PACG pre-clinical models, indicating that NO-GC-1–cGMP pathway is a strong candidate for therapeutic targeting.

## 2. Methods

### 2.1. Animals

All animal studies were conducted in accordance with the National

Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the IACUC committee at the Massachusetts General Hospital and Vanderbilt Medical Center. For RGC cultures, we obtained timed-pregnant Sprague-Dawley rats from (Charles River Laboratories, Wilmington, MA). Two – four days after birth, pups were collected, sacrificed by decapitation and retinas were processed for primary cultures of RGCs. For GC-1<sup>-/-</sup> studies, age-matched female wild type Sv/129S6 (WT) and GC-1<sup>-/-</sup> mice on an Sv/129S6 background were bred and housed at the Massachusetts General Hospital animal facility.(Buys et al., 2008) For microbead studies, age-matched (WT Sv/129S6) 8-week old female mice were bred in the animal facility at Massachusetts General Hospital. All mice were euthanized with 10 mg intraperitoneal (IP) pentobarbital injection. Observers masked to animal genotype, experimental group and diet performed all data acquisition and analyses described.

### 2.2. Microbead model

Microbead-induced IOP elevation was performed, as previously described.(Chen et al., 2011) WT mice were anesthetized with ketamine (100 mg/kg IP; Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (9 mg/kg; TranquiVed; Vedco, Inc., St. Joseph, MO). Additional analgesia was provided by topical application of tetracaine (1%; Bausch & Lomb, Tampa, FL). IOP elevation was induced unilaterally by 2  $\mu$ l injection of polystyrene microbeads (FluoSpheres; Invitrogen, Carlsbad, CA; 15- $\mu$ m diameter) into the anterior chamber of the right eye under a surgical microscope. Microbeads were formulated at  $5.0 \times 10^6$  beads/ml in sterile saline solution. The central cornea was gently punctured using a sharp 30-gauge needle (World Precision Instruments Inc., Sarasota, FL). Just prior to microbead injection, an air bubble was injected into the anterior chamber via a micropipette connected with a Hamilton syringe, coupled to a syringe pump to prevent excessive AqH egress from the eye. A small volume (2  $\mu$ l) of microbeads was then injected through the pre-formed anterior chamber entry site using the micropipette - Hamilton syringe-syringe pump equipment. Mice were placed on a heating pad for recovery after the injection, and a triple-antibiotic ointment (neomycin/polymyxin B/bacitracin) (Dechra Veterinary Products, Overland Park, KS) was applied topically onto the injected eye to prevent infection. In this study, microbeads were injected at Day 0 and Day 10 to maintain IOP elevation. In control groups, mice received 2  $\mu$ l injections of vehicle (saline) at Day 0 and Day 10.

### 2.3. IOP measurements

IOPs were measured at baseline, and post-injection at week 1, 2, 3 and 4 in the PACG model and at 2, 4, 6, 8 and 10 months after tadalafil treatment in the POAG model. IOP measurements were taken at the same time of day (between 1 and 3 PM). Mice were anesthetized by sequential isoflurane inhalation of 5% for 30 s, 3% for 1 min and then 2% for a further 1 min before measurements were taken. Isoflurane was delivered in 95% oxygen with a precision vaporizer and IOP measurements were taken after 2 min when animals lost toe pinch and blink reflex. IOPs were acquired with a TonoLab rebound tonometer (iCare, Franconia, NH, USA) after topical application of anesthesia (0.5% proparacaine hydrochloride). Five TonoLab final readings each were averaged to obtain a single IOP value per eye.

### 2.4. Retinal explants

For *ex vivo* retinal preparations, eyes were enucleated from WT mice and retinas rapidly removed and prepared as previously described.(Sappington et al., 2015) Briefly, explants were placed on organotypic culture inserts (0.4 mm pore; Millipore, Temecula, CA) held within culturing plates containing modified Neurobasal A media (2% B27 and 1% N2 supplements, 2 mM L-glutamine, 100  $\mu$ M inosine, 0.1%

gentamicin, 50 ng/ml BDNF, 20 ng/ml CNTF, and 10 ng/ml bFGF), and allowed to equilibrate overnight in an incubator at 37 °C with 5% CO<sub>2</sub>. Explants were maintained at ambient pressure in a standard incubator for 3 days and all experiments using explants were performed minimally in triplicate.

## 2.5. RGC cell separation and culture

Isolation of RGCs was carried out as previously described. (Sappington et al., 2006) Eyes ( $n \geq 16$ /preparation) from postnatal day 2 to 4 Sprague-Dawley rats were enucleated, retinas were dissected from each and stored on ice in Dulbecco modified Eagle medium plus 5% glucose (DMEM/glu; Gibco, Carlsbad, CA). Tissue was dissociated first by lightly vortexing for 10 s, and then by centrifugation ( $70 \times g$  for 6 min at 4 °C). Retinas were triturated by pipetting and incubated for 15 min at 37 °C in 1 mg/ml papain (Worthington, Lakewood, NJ) and 0.01% DNase I in Earle's Balanced Salt Solution. RGCs were purified by immunomagnetic separation, as previously described. (Shoge et al., 1999; Mukai et al., 2002; Levin, 2005) Briefly, to first remove Müller glia, the cell suspension was centrifuged (4 °C at  $250 \times g$ ) and re-suspended in DMEM/Glu with a polyclonal mouse anti-CD44 IgG (4 µg/ml, Catalog no: ab157107; Abcam). The suspension was incubated on ice for 10 min while shaking, before centrifugation (4 °C at  $250 \times g$ ) and incubation on ice for 15 min, shaking, with anti-mouse immunoglobulin G (IgG) secondary antibody conjugated to magnetic microbeads. The suspension was then loaded into a pre-equilibrated column in the presence of a magnetic field (Miltenyi Biotech, Auburn CA). To isolate RGCs, the remaining cell suspension was incubated with monoclonal mouse anti-Thy1.1/CD90 IgG on ice, while shaking for 10 min (5 µg/ml; Catalog no. 554895; BD Pharmingen, San Diego CA), and then for 15 min on ice, shaking, with antimouse IgG-labelled secondary and passed through a column in a magnetic field. The resulting Thy1.1/CD90-positive cells were plated into eight-chamber glass slides coated with laminin (0.01 mg/ml; Sigma) and grown in serum-free, B27-supplemented medium (NeuroBasal; Gibco), as previously described. (Sappington et al., 2006) The growth medium also contained 2 mM glutamine, 0.1% genomycin, 1% N<sub>2</sub> supplement (insulin 500 µg/ml; transferrin 10 mg/ml; progesterone 630 ng/ml; putrescine 1.6 mg/ml and selenite 520 ng/ml; Gibco), 50 ng/ml brain-derived neurotrophic factor (Invitrogen, Carlsbad, CA), 20 ng/ml ciliary neurotrophic factor (Invitrogen), 10 ng/ml bFGF (Invitrogen), and 100 µM inosine (Sigma). Before experiments, RGCs were maintained with the medium described above in a standard incubator containing 5% CO<sub>2</sub> until homeostasis was reached, as determined by neurite outgrowth and a stable level of viability (5–7 days).

## 2.6. Pharmacological interventions

For *in vivo* studies, food pellets containing cGMP-enhancing-compound (40 mg/kg tadalafil) were ordered from Research Diets, Inc. (New Brunswick, NJ, USA). All mice were fed with an *ad libitum* diet. The duration of tadalafil treatment was determined by the timeline of pathology in each model, such that tadalafil treatment was provided throughout pathogenesis and progression. For hemodynamic (WT) and cGMP (WT and GC-1<sup>-/-</sup>) studies described below, age-matched mice were fed tadalafil-containing chow (10 mg/kg/day) for 4 weeks prior to measurements. For microbead studies, WT mice were fed tadalafil-containing chow three days before the time of microbead injection, and then for 4 weeks until sacrifice and endpoints for the study were carried out. For chronic GC1<sup>-/-</sup> studies, tadalafil treatment began at maturity (3-month-old WT and GC-1<sup>-/-</sup> mice) and continued for 10 months until they were sacrificed. For *ex vivo* and *in vitro* studies, the cGMP analogue 8-Br-cGMP was used. For retinal explant experiments, explants were kept in culture for 24 h to equilibrate before incubation in the presence or absence of 8-Br-cGMP for another 48 h. Cell culture medium was then removed for LDH analysis (see below). Remaining

retinal tissue was then sonicated and protein extracted for western blot analysis (see below). For primary RGC cultures, cells were kept in culture for 5–7 days before addition of 8-Br-cGMP for an extra 48 h with or without 8-Br-cGMP.

## 2.7. cGMP quantification

Whole blood was collected in K<sub>2</sub>EDTA tubes (Becton Dickinson) from 3-month-old WT and GC-1<sup>-/-</sup> mice maintained on tadalafil chow for 4 weeks. Following centrifugation (4 °C for 15 min at  $1000 \times g$ ), serum was collected and snap-frozen in liquid nitrogen until analysis. cGMP concentrations were measured using the acetylation protocol of a cGMP ELISA Kit (Cayman Chemical) after sample dilution with supplied ELISA buffer as per manufacturer's specifications. Samples were standardized to protein concentration measured by BCA Protein Assay Kit (Pierce).

## 2.8. Hemodynamic measurements

Mean arterial pressure (MAP) was measured in 3-month-old WT mice maintained on tadalafil chow for 4 weeks. Mice were placed on a temperature-controlled surgical table with a rectal temperature probe for the maintenance of body temperature at 37 °C. The age-matched animals were anesthetized with 1.5 vol% isoflurane and 0.8 L/min O<sub>2</sub> flow. The right carotid artery was cannulated with a custom-made PE-10 catheter connected to a pressure transducer for monitoring of systemic blood pressure. After reaching a steady state, the MAP was recorded.

## 2.9. Retinal wholemount immunohistochemistry and RGC quantification

Eyes were enucleated, a small incision made in the cornea and post-fixed for 2 h in 4% paraformaldehyde (Boston Bioproducts, Ashland, MA) at 4 °C. Retinas were isolated from the eyecup and cuts made to create quadrants. Dissected retinas were blocked with 0.1% Triton-X100 and 2% BSA in PBS for 1 h, followed by incubation with the primary antibody β-III-tubulin, (1:400, 1 µg/ml, Millipore) overnight at 4 °C. The following day, retinas were washed  $3 \times$  in PBS and incubated for 2 h at room temperature with the secondary antibody anti-mouse Alexa Fluoro 594 (1:500, 4 µg/ml, Life Technologies). Finally, the 4',6-diamidino-2-phenylindole counterstain was added in PBS (1:1000) for 15 min at room temperature. Flat-mount stained retinas were placed on SuperFrost Plus slides (VWR, Batavia, IL) and coverslipped with mounting medium (VectaShield, Vector Lab, Burlingame, CA). Flat-mounted retina each with 4 unidirectional segments were imaged with a confocal microscope (Leica TSC SP5). For each retina, we employed a consistent sampling pattern that accounted for eccentricity whereby 16–20 non-overlapping images were taken from mid and peripheral regions of the retina (around 4–5 per unidirectional segment; at least two mid-central images and two peripheral per segment). The identity of each image was masked and anti-βIII-tubulin positive cells, denoting RGCs, were counted by two investigators, first by hand and then using CellProfiler and CellProfilerAnalyst software, as described previously. (Dordea et al., 2016) For each animal RGC counts were averaged and expressed as mean RGCs per mm<sup>2</sup> of retina.

## 2.10. Optic nerve axon quantification

Optic nerves were harvested and fixed in 2.5% Karnovsky fixative (2.5% formaldehyde/2.5% glutaraldehyde) at 4 °C. Semi-thin optic nerve cross sections (1 µm) were stained with 1% paraphenylenediamine (PPD, Fisher Scientific Co., Fair Lawn, NJ) and imaged on an upright microscope (Nikon bright-field microscope). Each section of optic nerve was imaged with 10–12 non-overlapping images taken per section at  $100 \times$ ; at least 5–6 central regions and 5–6 peripheral regions equidistant from the center of and circumference of the

nerve were taken. Quantification of myelinated axons on  $100\times$  images were carried out both manually and in ImageJ using the AxonJ plug-in by at least two masked investigators. AxonJ is an automated quantification tool used to count rodent axons on sections stained with PPD. (Zarei et al., 2016) The software uses an algorithm to detect axons and evaluates axon number in each section. To calculate axon density, axon counts were divided by the area counted ( $\text{mm}^2$ ). Axon density is expressed as mean axons/ $\text{mm}^2$ .

### 2.11. LDH cell death assay

After seeding, test compounds were prepared and added to primary RGC or retinal explants in culture medium. Culture medium was retained and LDH leakage measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Absorbances were measured at 490 nm using the SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices) and the SoftMax Pro Software (Molecular Devices). For data analysis, background absorbance was subtracted from sample absorbance. For every experiment 8 technical repeats of each sample were run, and the experiment repeated 3 times using a new retinal explant or isolation of primary cells.

### 2.12. Western blot analysis

Primary RGCs were collected from wells and centrifuged at (1200 relative centrifugal force for 5 min at  $4^\circ\text{C}$ ), washed twice in ice-cold PBS and the remaining pellet re-suspended in  $60\mu\text{l}$  radio-immunoprecipitation buffer (RIPA) buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, halt Protease inhibitor cocktail (ThermoFisher), and halt Phosphatase inhibitor cocktail (ThermoFisher). Cells were homogenized using an insulin syringe and vortexed for 3 s. Lysates were incubated for 20 min on ice and then centrifuged (15 min at  $>10,000\times g$ ). The supernatant was transferred into fresh Eppendorf tubes, snap frozen with liquid nitrogen, and stored at  $-80^\circ\text{C}$  if not immediately used. For retinal samples, each retina was placed into  $50\mu\text{l}$  RIPA buffer and homogenized by sonication (Fischer Scientific, FB50). Protein was measured in samples using the Pierce 660nn Protein Assay kit (ThermoFisher). Samples ( $20\mu\text{g}$  protein) were separated by SDS-PAGE on a 4–12% Bolt Bis-Tris gel (Invitrogen), electroblotted onto PVDF membranes and probed with the following primary antibodies: Cleaved Caspase-3 (9661S; 1:1000) Cell Signaling, Caspase-3 (9665S; 1:1000) cell signaling, and GAPDH (2118S; 1:1000) Cell Signaling. Proteins were detected using IRDye 680LT or IRDye 800CW secondary antibodies, Odyssey Blocking Buffer and a Li-Cor Odyssey Infrared Imaging System (Li-Cor Inc., Lincoln, NE) following manufacturer's protocol.

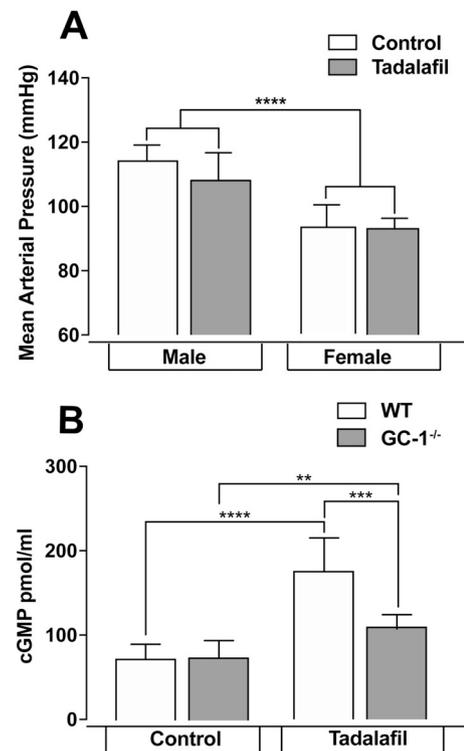
### 2.13. Statistical analysis

All data are presented as means  $\pm$  standard deviation (SD). GraphPad Prism 6 (La Jolla, CA) was used to perform statistical analyses of the data. For multiple comparisons in GC-1<sup>-/-</sup> and microbead studies, one-way ANOVA and the Welch's-corrected *t*-test were used. Mann-Whitney Rank Sum Tests were used for data sets that did not meet normality and/or equal variance requirements. A *p* value of 0.05 was considered significant.

## 3. Results

### 3.1. Tadalafil increases plasma cGMP in mice without elevating systemic blood pressure

Current indications for tadalafil treatment relate to its ability to increase smooth muscle relaxation in blood vessels via prolonged enhancement of cGMP levels. Since systemic blood pressure can affect AqH outflow and IOP, (Zhao et al., 2014; Lee et al., 2017) we first



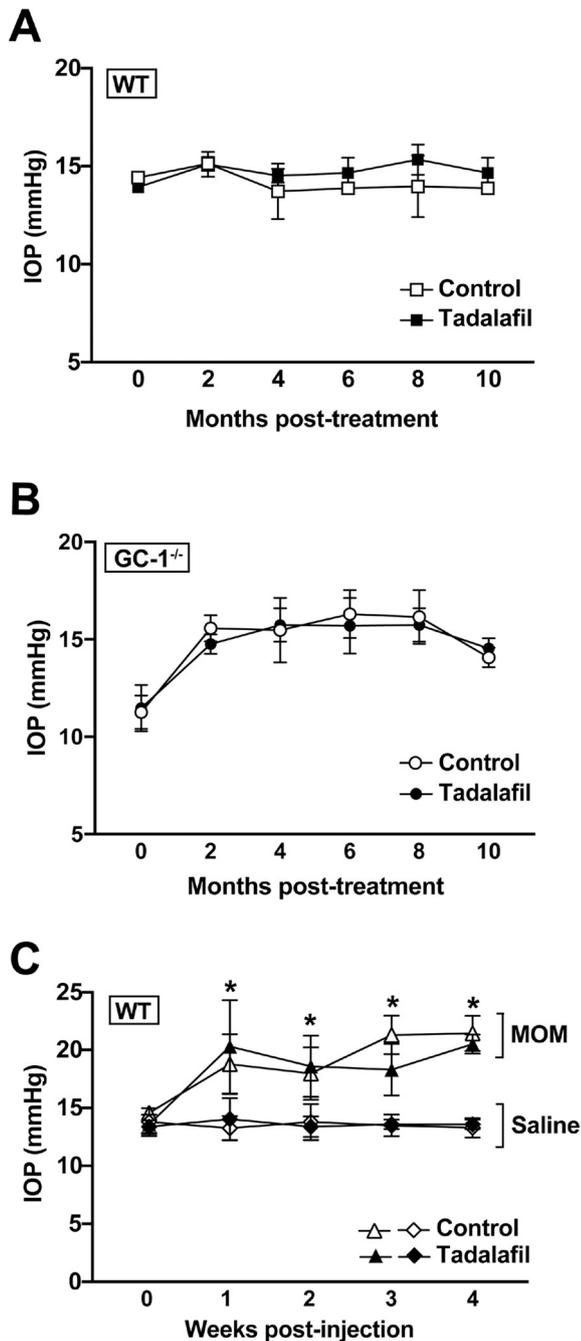
**Fig. 1.** Tadalafil does not alter MAP in male or female mice and increases plasma cGMP after 4 weeks. Naïve WT or GC-1<sup>-/-</sup> mice were maintained on 40 mg/Kg tadalafil chow for 4 weeks before MAP or cGMP measurements were taken,  $n = 7-9$  for all groups A). MAP does not change after tadalafil treatment, B) cGMP levels increase in both naïve WT and GC-1<sup>-/-</sup> mice. Data expressed as mean  $\pm$  S.D. \*\**p* = 0.004, \*\*\**p* = 0.0009 and \*\*\*\**p* < 0.00001.

confirmed that the dose of tadalafil in this study was beneath that required to induce systemic blood pressure effects. We maintained two-month old WT mice on control or tadalafil chow, dosed at 10 mg/kg/day for 4 weeks. Tadalafil treatment did not alter MAP in either male or female mice ( $p > 0.05$ ;  $n = 7$  for all groups, Fig. 1A), though MAP was 19% higher in male mice than in female mice, regardless of diet ( $p < 0.005$  for both control and tadalafil diets; Fig. 1A). This is a strain-dependent blood pressure phenomenon and has been reported previously, (Buys et al., 2012) and therefore only female mice were used throughout the remainder of the study.

To validate the ability of tadalafil to enhance cGMP levels, we measured cGMP in plasma of WT and GC-1<sup>-/-</sup> female mice fed a control or tadalafil diet. In mice maintained on control chow for 4 weeks, baseline cGMP levels were similar in GC-1<sup>-/-</sup> mice and WT mice (Fig. 1B). In mice treated with tadalafil, (4 weeks) plasma cGMP levels increased 2.4-fold and 1.5-fold in WT mice and GC-1<sup>-/-</sup> mice, respectively ( $p < 0.05$  for both;  $n = 9$  and  $n = 7$  respectively, Fig. 1B). cGMP levels in GC-1<sup>-/-</sup> maintained on tadalafil were 37% lower than tadalafil-treated WT mice ( $p = 0.0009$ ;  $n = 7$ , Fig. 1B). In addition to blood plasma, cGMP levels in retinal tissue was also analyzed; however, levels were below the minimal level of detection ( $< 20\text{ pmol cGMP/mg protein}$ ). Regardless, our results demonstrate target engagement of tadalafil and indicate that 4 weeks of daily dosing with tadalafil is sufficient to increase cGMP levels in both the presence and absence of GC-1 activity.

### 3.2. Tadalafil does not alter IOP in either murine model of glaucoma

Recent clinical trials demonstrate that a novel class of NO-donating drugs may be effective as IOP lowering agents for glaucoma. (Weinreb et al., 2015; Weinreb et al., 2016) To determine whether tadalafil treatment alters IOP, we measured IOP as a function of tadalafil



**Fig. 2.** Tadalafil does not affect IOP measurements in either POAG or PACG murine models of glaucoma. IOP was measured over time in WT or GC-1<sup>-/-</sup> mice in the POAG model of glaucoma, and in WT mice with and without microbead injection in the PACG model. A) IOP in WT mice does not change with tadalafil treatment, B) IOP in GC-1<sup>-/-</sup> mice does not change with tadalafil treatment and C) tadalafil treatment does not alter IOP in the PACG murine model of glaucoma in WT mice. Data expressed as mean  $\pm$  S.D. Statistical significances between saline and MOM (microbead occlusion model) groups are  $p < 0.0002$  for all comparisons.

treatment in GC-1<sup>-/-</sup> mice, a murine model of POAG and in WT mice using the microbead model of PACG. For POAG studies, we maintained GC-1<sup>-/-</sup> and WT mice on control or tadalafil diet for 10 months (beginning at 3 months of age) and measured IOP monthly. Comparisons between naïve WT and GC-1<sup>-/-</sup> mice determine the success of the POAG model and comparisons between GC-1<sup>-/-</sup> mice on control and tadalafil diets determines efficacy of tadalafil as an interventional treatment for POAG. For PACG, we maintained saline- and microbead-

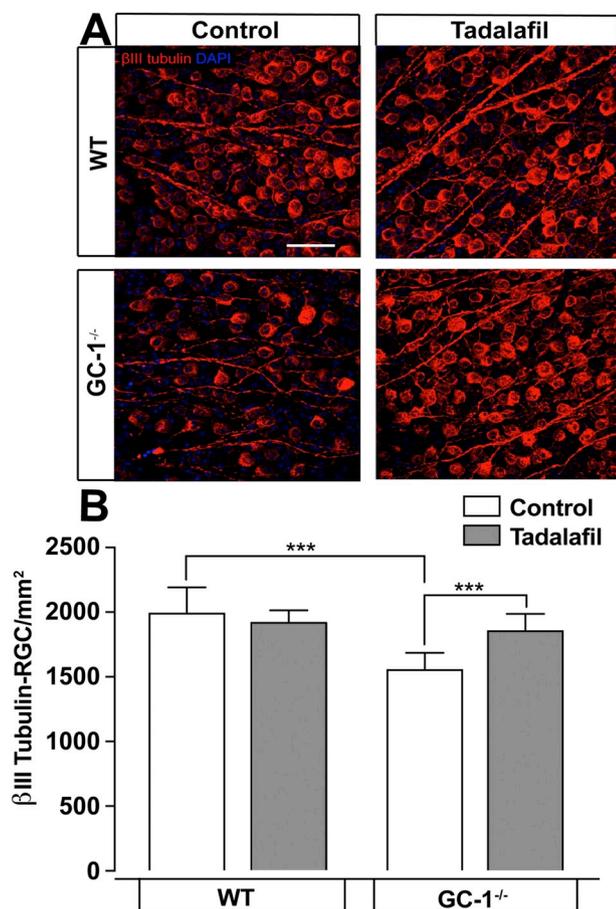
injected mice on control or tadalafil diet for 4 weeks (beginning 3 days prior to injection) and measured IOP three times per week for 4 weeks. Similarly, comparison between saline-injected and microbead-injected mice in the control diet group determines the success of the model. Comparison between control and tadalafil diet groups in microbead-injected mice determines the efficacy of tadalafil as an interventional treatment for PACG and finally, comparison of saline-injected animals determines baseline effects of tadalafil in healthy mice.

Treatment with tadalafil did not alter IOP in either WT (Fig. 2A) or GC-1<sup>-/-</sup> mice (Fig. 2B) over the 10-month treatment period ( $p > 0.05$  for all). In PACG studies, at its peak, IOP was 60% higher in microbead-injected eyes ( $21.7 \pm 0.8$  mmHg,  $n = 10$ ) than in saline-injected eyes ( $13.5 \pm 0.5$  mmHg,  $n = 7$ ) of mice maintained on control diet ( $p < 0.0001$ ; Fig. 2C). (Sappington et al., 2010) In tadalafil-treated mice, mean peak IOP was 51% higher in microbead-injected eyes ( $20.8 \pm 0.9$  mmHg,  $n = 9$ ) than in saline-injected eyes ( $13.8 \pm 0.9$  mmHg,  $n = 7$ ,  $p \leq 0.0002$ ; Fig. 2C). There was no significant difference in IOP between mice maintained on control and tadalafil diets for either saline- or microbead-injected eyes ( $p > .05$ , Fig. 2C). These data indicate that these tadalafil exposures did not significantly affect IOP elevation in either POAG or PACG murine models. Together, our findings suggest that daily administration of 10 mg/kg/day tadalafil enhances systemic cGMP levels without altering either systemic blood pressure or IOP.

### 3.3. Tadalafil protects RGCs in a murine model of POAG

Female GC-1<sup>-/-</sup> mice develop glaucoma characterized by age-dependent loss of RGCs and ON axons. (Buys et al., 2013) To investigate whether tadalafil can prevent optic neuropathy associated with GC-1 deficiency, age-matched WT and GC-1<sup>-/-</sup> mice were maintained on control or tadalafil chow for 10 months. After 10 months of treatment, we examined RGC degeneration by quantifying the density of  $\beta$ -tubulin-stained RGC soma in the retina and density of RGC axons in the optic nerve. Consistent with previous findings, (Buys et al., 2013)  $\beta$ tub + RGC density was 28% lower in GC-1<sup>-/-</sup> mice ( $1555 \pm 49.69$  RGC/mm<sup>2</sup>,  $n = 6$ ) than in WT mice ( $1991 \pm 76.71$  RGC/mm<sup>2</sup>,  $n = 6$ ) maintained on control diet ( $p = 0.0007$ ; Fig. 3A, B). In contrast, density of  $\beta$ tub + RGCs was 19% higher in retina from GC-1<sup>-/-</sup> mice maintained on tadalafil chow ( $1855 \pm 45.4$  RGC/mm<sup>2</sup>,  $n = 7$ ) than GC-1<sup>-/-</sup> mice maintained on control chow. This higher  $\beta$ tub + RGC density in GC-1<sup>-/-</sup> mice treated with tadalafil was similar to that in WT mice maintained on the tadalafil diet ( $1855 \pm 45.4$  RGC/mm<sup>2</sup>,  $n = 7$  vs.  $1918 \pm 36.61$  RGC/mm<sup>2</sup>,  $n = 5$ ,  $p = 0.34$  Fig. 3A, B). Tadalafil chow did not alter the density of  $\beta$ tub + RGCs in WT mice ( $1918 \pm 36.61$  RGC/mm<sup>2</sup>,  $n = 5$ ), as compared to control chow ( $1991 \pm 76.71$  RGC/mm<sup>2</sup>,  $n = 6$ ,  $p > 0.05$ ; Fig. 3A, B). These data indicate that tadalafil treatment preserves  $\beta$ tub + RGC soma in retina from a POAG murine model.

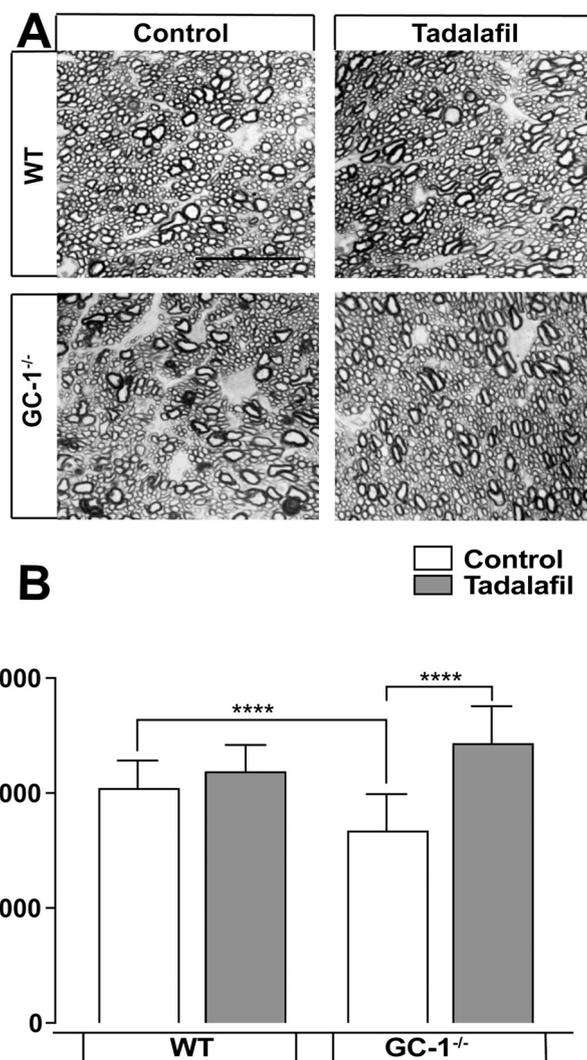
RGC degeneration in POAG occurs in a retrograde fashion, where RGC axons in the optic nerve degenerate prior to RGC soma in the retina. (Buckingham et al., 2008; Calkins, 2012) Thus, we quantified the RGC axon density in optic nerve from both control and GC-1<sup>-/-</sup> mice with and without tadalafil treatment at 10 months. In mice maintained on control chow, axon density in optic nerves from GC-1<sup>-/-</sup> mice ( $33,629 \pm 822.1$  axons/mm<sup>2</sup>,  $n = 6$ ) was 18% lower than in WT mice ( $41,016 \pm 540.2$  axons/mm<sup>2</sup>,  $n = 6$ ,  $p < 0.0001$ ; Fig. 4A, B). This is consistent with previously reported findings in this model. (Buys et al., 2013) Tadalafil treatment led to 7% higher axon counts in WT optic nerves ( $41,016 \pm 540.2$  axons/mm<sup>2</sup>,  $n = 6$  control chow vs. tadalafil chow at  $43,936 \pm 522.6$  axons/mm<sup>2</sup>,  $n = 5$ ,  $*p = 0.002$ ; Fig. 4B). In GC-1<sup>-/-</sup> mice, axons counts were 45% higher in mice fed tadalafil vs. control diet ( $48,828 \pm 707.4$  axons/mm<sup>2</sup>,  $n = 7$  vs.  $33,629 \pm 822.1$  axons/mm<sup>2</sup>,  $n = 6$ ,  $p < 0.0001$ ; Fig. 4B). These data indicate that tadalafil treatment markedly attenuates degeneration of RGC axons in the optic nerve of mice from a POAG murine model.



**Fig. 3.** Tadalafil prevents RGC loss in the murine POAG model of glaucoma. Retina were flatmounted and stained for  $\beta$ III tubulin. A) Representative  $\beta$ III tubulin-stained RGC micrographs in WT and GC-1<sup>-/-</sup> mice maintained on control or tadalafil chow for 10 months are shown, scale bar = 50 $\mu$ m. B) Quantitative analysis of the mean density of  $\beta$ III tubulin-positive RGCs (y-axis; mean RGCs/mm<sup>2</sup>  $\pm$  S.D) in retina reveals decreased RGC density in GC-1<sup>-/-</sup> mice that is reversed by tadalafil treatment. \*\*\*  $p < 0.0009$ . MOM = microbead occlusion model.

### 3.4. Tadalafil protects RGCs in a murine model of PACG

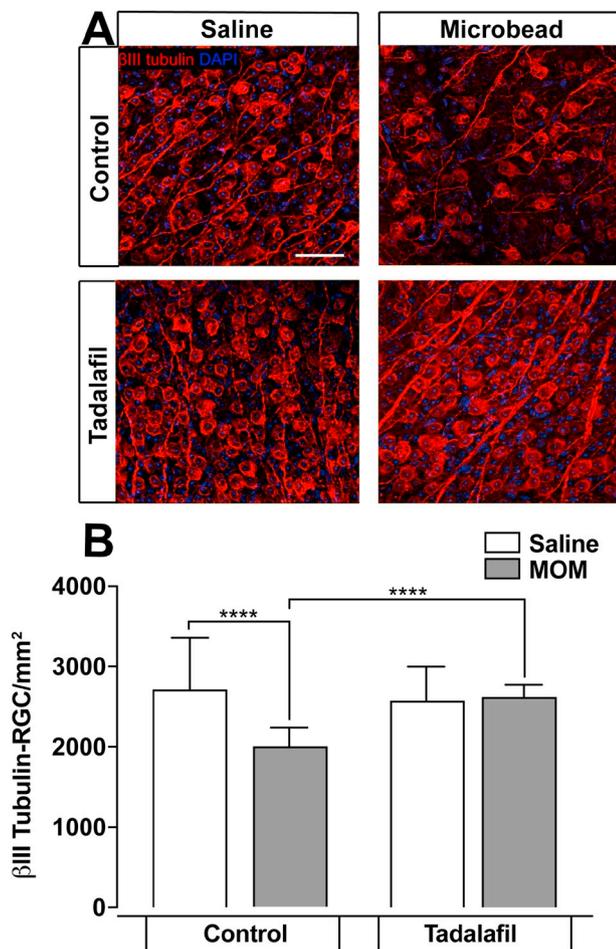
To determine whether the apparent neuroprotective properties of tadalafil are specific to POAG or could also translate to PACG-induced RGC degeneration, we examined the impact of tadalafil on RGC degeneration in the microbead occlusion model (MOM). (Sappington et al., 2010) In the MOM, intracameral injection of polystyrene microbeads leads to elevated IOP via blockade of aqueous drainage canals. (Mineo & Shaul, 2012) WT mice received intracameral injection of either microbeads or an equivalent volume of saline (experimental control). Three days prior to model induction, mice were fed tadalafil chow or control chow. After 4 weeks,  $\beta$ -tub+ RGC soma and axons were quantified in the retina and the optic nerve, respectively. Consistent with previous findings, (Sappington et al., 2010) microbead-induced ocular hypertension in mice maintained on control chow resulted in a 26% lower  $\beta$ -tub+ RGC density ( $2007 \pm 48.01$  RGC/mm<sup>2</sup>,  $n = 10$ ), as compared to saline-injected controls ( $2726 \pm 132$  RGC/mm<sup>2</sup>,  $n = 7$ ;  $p < 0.0001$ ; Fig. 5A, B). In contrast, the density of  $\beta$ -tub+ RGCs was similar in saline- ( $2580 \pm 86.9$  RGC/mm<sup>2</sup>,  $n = 7$ ) and microbead-injected eyes ( $2630 \pm 28.1$  RGC/mm<sup>2</sup>,  $n = 9$ ) from mice maintained on tadalafil diet ( $p > 0.05$ ; Fig. 5A, B). Across microbead-injected mice, the density of  $\beta$ -tub+ RGCs in microbead-injected eyes was 31% higher in tadalafil-treated mice than in mice maintained on control chow ( $2630 \pm 86.9$  RGC/mm<sup>2</sup>,  $n = 9$  vs.  $2007 \pm 48$  RGC/mm<sup>2</sup>,  $n = 10$ ,



**Fig. 4.** Tadalafil prevents ON axon depletion in WT and GC-1<sup>-/-</sup> mice in the murine POAG model of glaucoma. A) Representative paraphenylenediamine-stained ON axon micrographs in WT and GC-1<sup>-/-</sup> mice maintained on control or tadalafil chow, scale bar = 10 $\mu$ m. B) Quantitative analysis of the mean density of RGC axons (y-axis; mean axons/mm<sup>2</sup>  $\pm$  S.D) in optic nerve reveals decreased RGC axon density in GC-1<sup>-/-</sup> mice that is reversed by tadalafil treatment. \*\*\*\*,  $p < 0.0001$ .

$p < 0.0001$ , Fig. 5A, B). In contrast, there was no significant difference in the density of  $\beta$ -tub+ RGCs in saline-injected mice treated with tadalafil or maintained on control chow ( $2726 \pm 132$  RGC/mm<sup>2</sup>,  $n = 7$  vs.  $2580 \pm 86.9$  RGC/mm<sup>2</sup>,  $n = 7$ ,  $p > .05$ ; Fig. 5A, B).

In mice maintained on control chow, RGC axon density in optic nerves from microbead-injected eyes ( $33,563 \pm 1045$  axons/mm<sup>2</sup>,  $n = 10$ ) was 27% lower than that of saline-injected eyes ( $46,178 \pm 501.1$  axons/mm<sup>2</sup>,  $n = 7$ ,  $p < .0001$ ; Fig. 6A, B). Treatment with tadalafil prevented the loss of RGC axons: RGC axon density was similar in optic nerves from microbead-injected eyes ( $48,747 \pm 517.1$  axons/mm<sup>2</sup>,  $n = 9$ ) and saline-injected eyes ( $45,855 \pm 654.9$  axons/mm<sup>2</sup>,  $n = 7$  ( $p = 0.001$ ; Fig. 6A, B) and RGC axon density was 45% higher in mice treated with tadalafil chow ( $48,747 \pm 517.1$  axons/mm<sup>2</sup>,  $n = 9$ ) than in microbead-injected mice maintained on control chow ( $33,563 \pm 1045$  axons/mm<sup>2</sup>,  $n = 10$ ,  $p < .0001$ , Fig. 6A, B). RGC axon density did not differ significantly between optic nerves of saline-injected mice treated with tadalafil or maintained on control chow ( $45,855 \pm 654.9$  axons/mm<sup>2</sup>,  $n = 7$ , vs.  $46,178 \pm 501.1$  axons/mm<sup>2</sup>,  $n = 7$ ,  $p = 0.7$  Fig. 6A,B). Together, these data indicate that tadalafil treatment preserves  $\beta$ -tub+ RGC soma

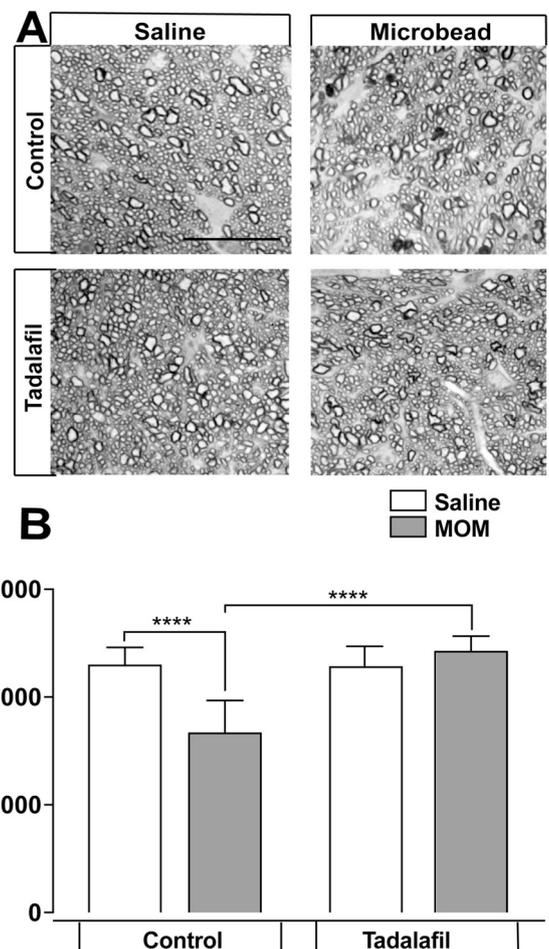


**Fig. 5.** Tadalafil prevents RGC loss in the murine PACG model of glaucoma. Retina were flatmounted and stained for  $\beta$ III tubulin. A) Representative  $\beta$ III tubulin-stained RGC micrographs in WT mice maintained on control or tadalafil chow for 1 month after saline or microbead injection are shown, scale bar = 50  $\mu$ M. B) Quantitative analysis of the mean density of  $\beta$ III tubulin-positive RGCs (y-axis; mean RGCs/mm<sup>2</sup>  $\pm$  S.D) in retina reveals decreased RGC density in mice injected with microbeads that is reversed by tadalafil treatment. \*\*\*  $p < 0.0001$ . MOM = microbead occlusion model.

in retina and RGC axon density in a PACG-like murine model.

### 3.5. cGMP directly influences necrotic and apoptotic cell death in retina

Our data indicate that daily administration of 10 mg/kg/day of tadalafil prevents RGC degeneration in both POAG (Figs. 3, 4) and PACG models (Figs. 5, 6), despite continued IOP elevation (Fig. 2). These results suggest that tadalafil influences RGC degeneration despite an IOP insult. Since tadalafil increases the bioavailability of cGMP, we sought to determine whether cGMP can directly impact the survival of RGCs. We examined necrotic and apoptotic cell death in organotypic culture of whole retina 72 h after axotomy in the presence (48 h) or absence of 100  $\mu$ M 8-Br-cGMP, a cell-membrane permeable cGMP analogue. Necrotic cell death was assessed by LDH assay and apoptotic death was assessed by caspase-3 cleavage (CC3). LDH release was 16% lower in whole retina explants treated with 8-Br-cGMP, than in those treated with vehicle ( $p = 0.02$ ; Fig. 7A). Similarly, CC3 cleavage was 38% lower in retina explants treated with 100  $\mu$ M 8-Br-cGMP than in those treated with vehicle ( $p = 0.002$ ; Fig. 7B). These data indicate that increased cGMP levels attenuate cell death of retinal tissue.



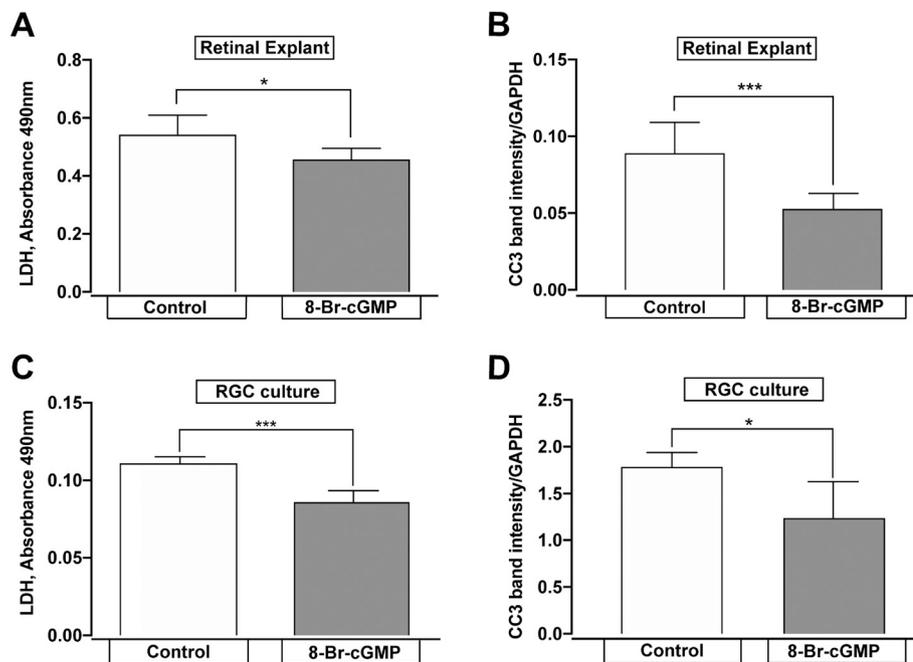
**Fig. 6.** Tadalafil prevents ON axon depletion in the murine PACG model of glaucoma. A) Representative PPD-stained ON axon micrographs in WT mice on control or tadalafil chow for 1 month after saline or microbead injection, scale bar = 10  $\mu$ M. B) Quantitative analysis of the mean density of RGC axons (y-axis; mean axons/mm<sup>2</sup>  $\pm$  S.D) in optic nerve reveals decreased RGC axon density in microbead-injected mice that is reversed by tadalafil treatment. Data expressed as mean  $\pm$  S.D. \*\*\*\*  $p < 0.0001$ . MOM = microbead occlusion model.

### 3.6. cGMP directly impacts necrotic and apoptotic cell death pathways in RGCs

Like organotypic culture, production of primary RGC cultures requires transection of RGC axons. Although there is some initial recovery from axotomy, cell survival depreciates with time in culture. This depreciation is attributable to both necrotic and apoptotic mechanisms. (Almasieh et al., 2012) Therefore we examined the ability of cGMP to impact post-axotomy cell death using primary cultures of purified rat RGCs. We treated RGC cultures with 100  $\mu$ M 8-Br-cGMP or vehicle for 48 h and measured baseline levels of necrotic and apoptotic cell death after 4 days in culture. Consistent with our results in whole retina explants, treatment with 8-Br-cGMP reduced LDH release by 22% ( $p = 0.0008$ ; Fig. 7C). Similarly, caspase-3 cleavage was also reduced by 30%, as compared to vehicle ( $p = 0.04$ ; Fig. 7D). These data indicate that increasing cGMP bioavailability improves survival of RGCs specifically and does so, in part, by mitigating both necrotic and apoptotic cell death.

## 4. Discussion

Multiple studies implicate impaired NO-cGMP signaling as a possible pathogenic mechanism in POAG. (Kang et al., 2010; Magalhaes da



**Fig. 7.** 8-Br-cGMP impedes necrotic and apoptotic pathways. A) Necrotic cell death, measured by LDH absorbance, was less pronounced in retinal explants incubated with 100  $\mu$ M 8-Br-cGMP than in explants incubated with vehicle,  $n = 6$ ,  $*p = 0.02$  B) Cleaved-caspase-3 (CC3) expression was attenuated in retinal explants when incubated with 100  $\mu$ M 8-Br-cGMP,  $n = 6$ ,  $**p = 0.002$ , C) Necrotic cell death, measured by LDH absorbance, was less pronounced in primary RGCs incubated with 100  $\mu$ M 8-Br-cGMP than in RGCs incubated with vehicle,  $n = 4$ ,  $***p = 0.0008$  and D) CC3 expression was attenuated in primary RGCs when incubated with 100  $\mu$ M 8-Br-cGMP,  $n = 4$ ,  $*p = 0.04$ . Data expressed as mean  $\pm$  S.D.

Silva et al., 2012; Doganay et al., 2002; Galassi et al., 2004; Wareham et al., 2018) It is known that NO and cGMP regulate AqH outflow and IOP in pre-clinical models and clinical models (Ellis et al., 2009; Khoobehi et al., 2011; Kotikoski et al., 2003; Krauss et al., 2011; Weinreb et al., 2015; Weinreb et al., 2016) and that NO has been attributed to both neurotoxic and neuroprotective roles, which appear to stem from concentration effects *in vivo*. Excess endogenous NO is noxious, since it can undergo oxidative reduction reactions to produce reactive nitrogen species (Pacher et al., 2007; Guix et al., 2005) which have been implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's Disease (Sobrevia et al., 2016) However, several studies have revealed neuroprotective properties of NO through its downstream activation of the cGMP pathway and subsequent downstream effectors, such as protein kinases and  $Ca^{2+}$  channels (Kohgami et al., 2010; Ditlevsen et al., 2007; Thippeswamy et al., 2005) Our previous work established female GC-1<sup>-/-</sup> mice as a new animal model for POAG, suggesting a central role for NO/GC-1 signaling in the regulation of IOP and RGC homeostasis (Buys et al., 2013)

Despite an important role for the NO-cGMP pathway emerging in the pathophysiology of glaucoma (Wareham et al., 2018) there have been little preclinical work done to explore the therapeutic potential of harnessing this pathway in glaucoma treatment. We show in two murine models of glaucoma (POAG and PACG) that systemic delivery of tadalafil prevents IOP-induced degeneration of RGCs. This apparent neuroprotection was independent of IOP, as we detected no change in the magnitude or duration of IOP elevation with tadalafil treatment in either the POAG or PACG model. There are some reports that the PDE5 inhibitors tadalafil and sildenafil cause transient increases in IOP in sheep (Gerometta et al., 2010) and also in humans (Gerometta et al., 2011) However, our results align well with a long-term study in humans with POAG that demonstrated no significant change in IOP when sildenafil, also a PDE5 inhibitor, was administered at a dose of 100 mg (Grunwald et al., 2001)

While tadalafil treatment did not impact IOP elevation in our POAG and PACG models, systemic enhancement of cGMP has the potential to affect other relevant parameters, such as blood pressure. We determined that our treatment paradigm for tadalafil was sufficient to enhance systemic cGMP levels, illustrating target engagement, without elevating systemic blood pressure, a potential confounding aspect discussed below. It may seem surprising that genetic ablation of GC-1 is

not associated with reduced cGMP levels in mice maintained on control chow; however, in addition to GC-1, cGMP is generated by other guanylyl cyclase isoforms, such as GC-2 and natriuretic peptide receptors (Lucas et al., 2000) Despite this, plasma cGMP levels were lower in GC-1<sup>-/-</sup> mice than WT mice fed tadalafil diet, confirming that ablation of GC-1 is associated with impaired cGMP signaling.

Given that tadalafil treatment in our study was sufficient to enhance cGMP levels and protect RGCs independently of IOP, we explored whether cGMP enhancement directly impacted survival of retinal cells generally and RGCs specifically. In *ex vivo* retinal explants, incubation with 8-Br-cGMP led to a decrease in extracellular LDH, an easily quantifiable indicator of necrotic cell death. Levels of CC3 were also lower in tadalafil than in vehicle-treated explants, suggesting a reduction in the activation of pro-apoptotic pathways. *In vitro* studies in primary purified RGCs yielded similar results, indicating that enhancement of cGMP levels has the potential to mitigate both necrotic and apoptotic pathways of cell death in retina and more specifically, RGCs. Together, these data suggest that the apparent neuroprotective effect of tadalafil in our POAG and PACG models arises, at least in part from cGMP-dependent attenuation of pro-apoptotic pathways, known to underlie RGC degeneration in both murine glaucoma models (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Garcia-Valenzuela et al., 1995; Quigley et al., 1995; Reichstein et al., 2007) and in human patients (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Garcia-Valenzuela et al., 1995; Quigley et al., 1995; Reichstein et al., 2007) Consistent with our findings in primary RGCs, anti-apoptotic roles of NO and cGMP have already been implicated in RGC survival (Olivares-Gonzalez et al., 2016; Schallner et al., 2013) and in other neuronal cell lines, such as motor and sympathetic neurons (Farinelli et al., 1996; Estevez et al., 1998) and hippocampal neurons (Barger et al., 1995)

The encouraging results reported in this study are likely to trigger additional research. Firstly, to show a true neuroprotective role of tadalafil in glaucoma, assessment of the effect of tadalafil on visual function in the glaucoma models studied will be required. Also, while no effect of 6-month treatments with tadalafil or sildenafil on electroretinography was detected in healthy humans (Cordell et al., 2009) chronic tadalafil treatment was associated with toxic effects on photoreceptors in rat studies (Sarhan & Omar, 2018) Thus, studies of the effects of chronic tadalafil on ultrastructural features should be

executed when further exploring a potential therapeutic role of tadalafil in glaucoma management. Any side effects of the use of PDE5 inhibitors may be circumvented by taking advantage of other compounds under clinical development that increase cGMP bioavailability (Buys et al., 2018). In addition, other dosing strategies for tadalafil and other PDE inhibitors have not yet been explored, and were beyond the scope of this study. Other PDE5 inhibitors have been used in the clinic and previous studies have shown that they are effective as neuroprotective agents. In a rat model of hypoxic ischemia, activation of the NO-cGMP pathway by sildenafil, a PDE5 inhibitor similar to tadalafil but with reduced half-life and specificity, reduced apoptosis, astrocytosis and microgliosis in the brain (Charriaut-Marlangue et al., 2014). We selected the PDE5 inhibitor tadalafil to elevate cGMP levels *in vivo* due to its longer half life (Coward & Carson, 2008) proven safety in chronic treatments (Forge et al., 2006) and ability to cross the blood-brain barrier (Garcia-Barroso et al., 2013). Tadalafil has also been proven to be neuroprotective in spinal cord injury (Serarlsan et al., 2010) and in ischemia/reperfusion injury in the fetal rat brain (Ozdegirmenci et al., 2011).

Visual disturbances have been reported in patients taking PDE5 inhibitors (Stockman et al., 2007; Center for Drug Evaluation and Research, 1998) the most common being an increased sensitivity to light (Carter, 2007; Cunningham & Smith, 2001; Santaella & Fraunfelder, 2007). These symptoms likely arise from off-target inhibition of PDE6 in the retina. However, the selectivity of PDE5 over PDE6 is 700-fold for tadalafil (CIALISR, 2008) compared with 10-fold for sildenafil (ViagraR, 2008) and 15-fold for vardenafil (LEVITRAN, 2008) so off-target effects when using tadalafil are likely limited. In fact, the rate of occurrence for these symptoms is low at only 0.1% of tadalafil users (Brock et al., 2002). The decision to dose tadalafil so as not to impact MAP was to prevent the potentially confounding effect of MAP on ocular blood flow. Although we did not identify changes in MAP and there are conflicting data on the role of systemic blood pressure in glaucoma etiology and progression (Bonomi et al., 2000; Mitchell et al., 2004; Orzalessi et al., 2007; Leske, 2009) we cannot rule out a tadalafil-mediated effect on retinal vascular function as a contributor to the apparent neuroprotection we noted *in vivo*.

## 5. Conclusions

Overall, our results indicate that increasing cGMP bioavailability promotes RGC survival in murine models of POAG and PACG, likely through direct modulation of pro- and anti-apoptotic pathways. Furthermore, our data identify tadalafil and related compounds that enhance cGMP bioavailability and that are already clinically available or being tested in clinical trials for a variety of indications (Buys et al., 2018) as potential therapeutics for direct RGC neuroprotection. Such therapeutics could serve to increase efficacy of treatment in glaucoma patients with continued disease progression despite IOP lowering interventions. Further experiments are warranted to explore the possible effects of tadalafil on retinal vasculature and other putative mechanisms of RGC survival in glaucoma models.

## Funding sources

These studies were supported by the National Eye Institute awards R01EY022746 (ESB), R01EY020496 (RMS), R01EY015473 (LRP) and P30EY08126 (Vanderbilt Vision Research Center), Unrestricted (Vanderbilt Eye Institute) awards from Research to Prevent Blindness, Inc.

## References

Almasieh, M., Wilson, A.M., Morquette, B., Cueva Vargas, J.L., Di Polo, A., 2012. The molecular basis of retinal ganglion cell death in glaucoma. *Prog. Retin. Eye Res.* 31, 152–181.

The effectiveness of intraocular pressure reduction in the treatment of normal-tension glaucoma. *Am J. Ophthalmol.* 126, 498–505.

Awadalla, M.S., Thapa, S.S., Hewitt, A.W., JE, Craig, Burdon, K.P., 2013. Association of eNOS polymorphisms with primary angle-closure glaucoma. *Invest. Ophthalmol. Vis. Sci.* 54, 2108–2114.

Ayub, H., Khan, M.I., Micheal, S., Akhtar, F., Ajmal, M., Shafique, S., Ali, S.H., den Hollander, A.I., Ahmed, A., Qamar, R., 2010. Association of eNOS and HSP70 gene polymorphisms with glaucoma in Pakistani cohorts. *Mol. Vis.* 16, 18–25.

Barger, S.W., Fiscus, R.R., Ruth, P., Hofmann, F., Mattson, M.P., 1995. Role of cyclic GMP in the regulation of neuronal calcium and survival by secreted forms of beta-amyloid precursor. *J. Neurochem.* 64, 2087–2096.

Berkelaar, M., Clarke, D.B., Wang, Y.C., Bray, G.M., Aguayo, A.J., 1994. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J. Neurosci.* 14, 4368–4374.

Bonomi, L., Marchini, G., Marraffa, M., Bernardi, P., Morbio, R., Varotto, A., 2000. Vascular risk factors for primary open angle glaucoma: the Egna-Neumarkt Study. *Ophthalmology* 107, 1287–1293.

Brock, G.B., McMahon, C.G., Chen, K.K., Costigan, T., Shen, W., Watkins, V., Anglin, G., Whitaker, S., 2002. Efficacy and safety of tadalafil for the treatment of erectile dysfunction: results of integrated analyses. *J. Urol.* 168, 1332–1336.

Buckingham, B.P., Inman, D.M., Lambert, W., Oglesby, E., Calkins, D.J., Steele, M.R., Vetter, M.L., Marsh-Armstrong, N., Horner, P.J., 2008. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J. Neurosci.* 28, 2735–2744.

Buys, E.S., Sips, P., Vermeersch, P., Raheer, M.J., Rogge, E., Ichinose, F., Dewerchin, M., Bloch, K.D., Janssens, S., Brouckaert, P., 2008. Gender-specific hypertension and responsiveness to nitric oxide in sGC $\alpha$ 1 knockout mice. *Cardiovasc. Res.* 79, 179–186.

Buys, E.S., Raheer, M.J., Kirby, A., Mohd, S., Baron, D.M., Hayton, S.R., Tainsh, L.T., Sips, P.Y., Rauwerdink, K.M., Yan, Q., Tainsh, R.E., Shakartzi, H.R., Stevens, C., Decaluwe, K., Rodrigues-Machado Mda, G., Malhotra, R., f, Wang, T., Brouckaert, P., Daly, M.J., Bloch, K.D., 2012. Genetic modifiers of hypertension in soluble guanylate cyclase  $\alpha$ 1-deficient mice. *J. Clin. Invest.* 122, 2316–2325.

Buys, E.S., Ko, Y.C., Alt, C., Hayton, S.R., Jones, A., Tainsh, L.T., Ren, R., Giani, A., Clerle, M., Abernathy, E., Tainsh, R.E., Oh, D.J., Malhotra, R., Arora, P., de Waard, N., Yu, B., Turcotte, R., Nathan, D., Scherrer-Crosbie, M., Loomis, S.J., Kang, J.H., Lin, C.P., Gong, H., Rhee, D.J., Brouckaert, P., Wiggs, J.L., Gregory, M.S., Pasquale, L.R., Bloch, K.D., Ksander, B.R., 2013. Soluble guanylate cyclase  $\alpha$ 1-deficient mice: A novel murine model for primary open angle glaucoma. *PLoS One* 8, e60156.

Buys, E.S., Zimmer, D.P., Chickering, J., Graul, R., Chien, Y.T., Profy, A., Haddock, J.R., Masferrer, J.L., Milne, G.T., 2018. Discovery and development of next generation sGC stimulators with diverse multidimensional pharmacology and broad therapeutic potential. *Nitric Oxide* 78, 72–80.

Calkins, D.J., 2012. Critical pathogenic events underlying progression of neurodegeneration in glaucoma. *Prog. Retin. Eye Res.* 31, 702–719.

Carter, J.E., 2007. Anterior ischemic optic neuropathy and stroke with use of PDE-5 inhibitors for erectile dysfunction: cause or coincidence? *J. Neurol. Sci.* 262, 89–97.

Center for Drug Evaluation and Research, 1998. Study 148–232. A Randomised, Double-Blind, Placebo-Controlled, Crossover Pilot Study to Investigate the Effects of a Single Oral Tablet Dose of Sildenafil (200 mg) on Visual Function (Electroretinogram, Photostress, Visual Field and Colour Discrimination Tests) in Healthy Male Volunteers and Patients with Diabetic Retinopathy. *Viagra (Sildenafil): “Joint Clinical Review” for NDA-20-895.* Center for Drug Evaluation and Research, Food and Drug Administration, Washington, DC, pp. 177–178.

Chang, J.Y., Stamer, W.D., Bertrand, J., Read, A.T., Marando, C.M., Ethier, C.R., Overby, D.R., 2015. Role of nitric oxide in murine conventional outflow physiology. *Am. J. Phys. Cell Phys.* 309, C205–C214.

Charriaut-Marlangue, C., Nguyen, T., Bonnin, P., Duy, A.P., Leger, P.L., Csaba, Z., Pansiot, J., Bourgeois, T., Renolleau, S., Baud, O., 2014. Sildenafil mediates blood-flow redistribution and neuroprotection after neonatal hypoxia-ischemia. *Stroke* 45, 850–856.

Chen, H., Wei, X., Cho, K.S., Chen, G., Sappington, R., Calkins, D.J., Chen, D.F., 2011. Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. *Invest. Ophthalmol. Vis. Sci.* 52, 36–44.

CIALISR, 2008. Full Prescribing Information Tadalafil. Lilly ICOS, LLC, Indianapolis, IN.

Cordell, W.H., Maturi, R.K., Costigan, T.M., Marmor, M.F., Weleber, R.G., Coupland, S.G., Danis, R.P., McGittigan Jr., J.W., Antoszyk, A.N., Klise, S., Sides, G.D., Consortium ERGTDPCIA, 2009. Retinal effects of 6 months of daily use of tadalafil or sildenafil. *Arch. Ophthalmol.* 127, 367–373.

Coward, R.M., Carson, C.C., 2008. Tadalafil in the treatment of erectile dysfunction. *Ther. Clin. Risk Manag.* 4, 1315–1330.

Cunningham, A.V., Smith, K.H., 2001. Anterior ischemic optic neuropathy associated with viagra. *J. Neuroophthalmol.* 21, 22–25.

Ditlevsen, D.K., Kohler, L.B., Berezin, V., Bock, E., 2007. Cyclic guanosine monophosphate signalling pathway plays a role in neural cell adhesion molecule-mediated neurite outgrowth and survival. *J. Neurosci. Res.* 85, 703–711.

Doganay, S., Evereklioglu, C., Turkoz, Y., Er, H., 2002. Decreased nitric oxide production in primary open-angle glaucoma. *Eur. J. Ophthalmol.* 12, 44–48.

Dordea, A.C., Bray, M.A., Allen, K., Logan, D.J., Fei, F., Malhotra, R., Gregory, M.S., Carpenter, A.E., Buys, E.S., 2016. An open-source computational tool to automatically quantify immunolabeled retinal ganglion cells. *Exp. Eye Res.* 147, 50–56.

Ellis, D.Z., Dismuke, W.M., Chokshi, B.M., 2009. Characterization of soluble guanylate cyclase in NO-induced increases in aqueous humor outflow facility and in the trabecular meshwork. *Invest. Ophthalmol. Vis. Sci.* 50, 1808–1813.

Emam, W.A., Zidan, H.E., Abdulhalim, B.E., Dabour, S.A., Ghali, M.A., Kamal, A.T., 2014. Endothelial nitric oxide synthase polymorphisms and susceptibility to high-tension

- primary open-angle glaucoma in an Egyptian cohort. *Mol. Vis.* 20, 804–811.
- Estevez, A.G., Spear, N., Thompson, J.A., Cornwell, T.L., Radi, R., Barbeito, L., Beckman, J.S., 1998. Nitric oxide-dependent production of cGMP supports the survival of rat embryonic motor neurons cultured with brain-derived neurotrophic factor. *J. Neurosci.* 18, 3708–3714.
- Farinelli, S.E., Park, D.S., Greene, L.A., 1996. Nitric oxide delays the death of trophic factor-deprived PC12 cells and sympathetic neurons by a cGMP-mediated mechanism. *J. Neurosci.* 16, 2325–2334.
- Forgue, S.T., Patterson, B.E., Bedding, A.W., Payne, C.D., Phillips, D.L., Wrishko, R.E., Mitchell, M.I., 2006. Tadalafil pharmacokinetics in healthy subjects. *Br. J. Clin. Pharmacol.* 61, 280–288.
- Friedman, D.S., Wolfs, R.C., O'Colmain, B.J., Klein, B.E., Taylor, H.R., West, S., Leske, M.C., Mitchell, P., Congdon, N., Kempen, J., 2004. Eye Diseases Prevalence Research G. Prevalence of open-angle glaucoma among adults in the United States. *Arch. Ophthalmol.* 122, 532–538.
- Galassi, F., Renieri, G., Sodi, A., Ucci, F., Vannozzi, L., Masini, E., 2004. Nitric oxide proteins and ocular perfusion pressure in primary open angle glaucoma. *Br. J. Ophthalmol.* 88, 757–760.
- García-Barroso, C., Ricobaraza, A., Pascual-Lucas, M., Unceta, N., Rico, A.J., Goicolea, M.A., Salles, J., Lanciego, J.L., Oyarzabal, J., Franco, R., Cuadrado-Tejedor, M., García-Osta, A., 2013. Tadalafil crosses the blood-brain barrier and reverses cognitive dysfunction in a mouse model of AD. *Neuropharmacology* 64, 114–123.
- García-Valenzuela, E., Gorczyca, M., Darzynkiewicz, Z., Sharma, S.C., 1994. Apoptosis in adult retinal ganglion cells after axotomy. *J. Neurobiol.* 25, 431–438.
- García-Valenzuela, E., Shareef, S., Walsh, J., Sharma, S.C., 1995. Programmed cell death of retinal ganglion cells during experimental glaucoma. *Exp. Eye Res.* 61, 33–44.
- Garway-Heath, D.F., Crabb, D.P., Bunce, C., Lascaratos, G., Amalfitano, F., Anand, N., Azuara-Blanco, A., Bourne, R.R., Broadway, D.C., Cunliffe, I.A., Diamond, J.P., Fraser, S.G., Ho, T.A., Martin, K.R., McNaught, A.I., Negi, A., Patel, K., Russell, R.A., Shah, A., Spry, P.G., Suzuki, K., White, E.T., Wormald, R.P., Xing, W., Zeyen, T.G., 2015. Latanoprost for open-angle glaucoma (UKGTS): a randomised, multicentre, placebo-controlled trial. *Lancet* 385, 1295–1304.
- Gerometta, R., Alvarez, L.J., Candia, O.A., 2010. Effects of sildenafil and tadalafil on intraocular pressure in sheep: implications for aqueous humor dynamics. *Invest. Ophthalmol. Vis. Sci.* 51, 3139–3144.
- Gerometta, R., Alvarez, L.J., Candia, O.A., 2011. Effect of sildenafil citrate on intraocular pressure and blood pressure in human volunteers. *Exp. Eye Res.* 93, 103–107.
- Grunwald, J.E., Jacob, S.S., Siu, K., Piltz, J., Dupont, J., 2001. Acute effects of sildenafil citrate (Viagra) on intraocular pressure in open-angle glaucoma. *Am J. Ophthalmol.* 132, 872–874.
- Guix, F.X., Uribealago, I., Coma, M., Munoz, F.J., 2005. The physiology and pathophysiology of nitric oxide in the brain. *Prog. Neurobiol.* 76, 126–152.
- Heijl, A., Leske, M.C., Bengtsson, B., Hyman, L., Bengtsson, B., Hussein, M., 2002. Early Manifest Glaucoma Trial G. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial. *Arch. Ophthalmol.* 120, 1268–1279.
- Jeoung, J.W., Kim, D.M., Oh, S., Lee, J.S., Park, S.S., Kim, J.Y., 2017. The relation between endothelial nitric oxide synthase polymorphisms and normal tension glaucoma. *J. Glaucoma* 26, 1030–1035.
- Kang, J.H., Wiggs, J.L., Rosner, B.A., Hankinson, S.E., Abdrabou, W., Fan, B.J., Haines, J., Pasquale, L.R., 2010. Endothelial nitric oxide synthase gene variants and primary open-angle glaucoma: interactions with sex and postmenopausal hormone use. *Invest. Ophthalmol. Vis. Sci.* 51, 971–979.
- Kang, J.H., Wiggs, J.L., Haines, J., Abdrabou, W., Pasquale, L.R., 2011. Reproductive factors and NOS3 variant interactions in primary open-angle glaucoma. *Mol. Vis.* 17, 2544–2551.
- Kass, M.A., Heuer, D.K., Higginbotham, E.J., Johnson, C.A., Keltner, J.L., Miller, J.P., Parrish 2nd, R.K., Wilson, M.R., Gordon, M.O., 2002. The ocular hypertension treatment study: a randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch. Ophthalmol.* 120, 701–713.
- Kawase, K., Vittitow, J.L., Weinreb, R.N., Araie, M., Group JS. Long-term Safety, 2016. Efficacy of latanoprostene bunod 0.024% in Japanese subjects with open-angle glaucoma or ocular hypertension: the JUPITER study. *Adv. Ther.* 33, 1612–1627.
- Khawaja, A.P., Cooke Bailey, J.N., Wareham, N.J., Scott, R.A., Simcoe, M., Igo Jr., R.P., Song, Y.E., Wojciechowski, R., Cheng, C.Y., Khaw, P.T., Pasquale, L.R., Haines, J.L., Foster, P.J., Wiggs, J.L., Hammond, C.J., Hysi, P.G., Eye, U.K.B., Vision, C., Consortium N., 2018. Genome-wide analyses identify 68 new loci associated with intraocular pressure and improve risk prediction for primary open-angle glaucoma. *Nat. Genet.* 50, 778–782.
- Khoobehi, B., Chiroli, V., Ronchetti, D., Miglietta, D., Thompson, H., Ongini, E., Impagnatiello, F., 2011. Enhanced oxygen saturation in optic nerve head of non-human primate eyes following the intravitreal injection of NCX 434, an innovative nitric oxide-donating glucocorticoid. *J. Ocul. Pharmacol. Ther.* 27, 115–121.
- Kohgami, S., Ogata, T., Morino, T., Yamamoto, H., Schubert, P., 2010. Pharmacological shift of the ambiguous nitric oxide action from neurotoxicity to cyclic GMP-mediated protection. *Neurosci. Res.* 32, 938–944.
- Kotlikoski, H., Vapaatalo, H., Oksala, O., 2003. Nitric oxide and cyclic GMP enhance aqueous humor outflow facility in rabbits. *Curr. Eye Res.* 26, 119–123.
- Krauss, A.H., Impagnatiello, F., Toris, C.B., Gale, D.C., Prasanna, G., Borghi, V., Chiroli, V., Chong, W.K., ST, Carreiro, Ongini, E., 2011. Ocular hypotensive activity of BOL-303259-X, a nitric oxide donating prostaglandin F<sub>2</sub>alpha agonist, in preclinical models. *Exp. Eye Res.* 93, 250–255.
- Lee, N.Y., Jung, Y., Han, K., Park, C.K., 2017. Fluctuation in systolic blood pressure is a major systemic risk factor for development of primary open-angle glaucoma. *Sci. Rep.* 7.
- Lei, Y., Zhang, X., Song, M., Wu, J., Sun, X., 2015. Aqueous humor outflow physiology in NOS3 knockout mice. *Invest. Ophthalmol. Vis. Sci.* 56, 4891–4898.
- Leske, M.C., 2009. Ocular perfusion pressure and glaucoma: clinical trial and epidemiologic findings. *Curr. Opin. Ophthalmol.* 20, 73–78.
- Levin, L.A., 2005. Retinal ganglion cells and supporting elements in culture. *J. Glaucoma* 14, 305–307.
- LEVITRAN, 2008. Full Prescribing Information Vardenafil. Bayer Pharmaceuticals Corporation, West Haven, CT.
- Lucas, K.A., Pitari, G.M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., KP, Chepenik, Waldman, S.A., 2000. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev.* 52, 375–414.
- Mabuchi, F., Aihara, M., Mackey, M.R., Lindsey, J.D., Weinreb, R.N., 2003. Optic nerve damage in experimental mouse ocular hypertension. *Invest. Ophthalmol. Vis. Sci.* 44, 4321–4330.
- Magalhaes da Silva, T., Rocha, A.V., Lacchini, R., Marques, C.R., Silva, E.S., Tanus-Santos, J.E., Rios-Santos, F., 2012. Association of polymorphisms of endothelial nitric oxide synthase (eNOS) gene with the risk of primary open angle glaucoma in a Brazilian population. *Gene* 502, 142–146.
- Mineo, C., Shaul, P.W., 2012. Regulation of eNOS in caveolae. *Adv. Exp. Med. Biol.* 729, 51–62.
- Mitchell, P., Lee, A.J., Rochtchina, E., Wang, J.J., 2004. Open-angle glaucoma and systemic hypertension: the blue mountains eye study. *J. Glaucoma* 13, 319–326.
- Muenster, S., Lieb, W.S., Fabry, G., Allen, K.N., Kamat, S.S., Guy, A.H., Dordea, A.C., Teixeira, L., Tainsh, R.E., Yu, B., Zhu, W., Ashpole, N.E., Malhotra, R., Brouckaert, P., Bloch, D.B., Scherrer-Crosbie, M., Stamer, W.D., Kuehn, M.H., LR, Pasquale, Buys, E.S., 2017. The ability of nitric oxide to lower intraocular pressure is dependent on guanylyl cyclase. *Invest. Ophthalmol. Vis. Sci.* 58, 4826–4835.
- Mukai, S., Mishima, H.K., Shoge, K., Shinya, M., Ishihara, K., Sasa, M., 2002. Existence of ionotropic glutamate receptor subtypes in cultured rat retinal ganglion cells obtained by the magnetic cell sorter method and inhibitory effects of 20-hydroxyecdysone, a neurosteroid, on the glutamate response. *Jpn. J. Pharmacol.* 89, 44–52.
- Nunes, H.F., Ananina, G., Costa, V.P., Zanchin, N.I.T., de Vasconcelos, J.P.C., de Melo, M.B., 2018. Investigation of CAV1/CAV2 rs4236601 and CDKN2B-AS1 rs2157719 in primary open-angle glaucoma patients from Brazil. *Ophthalmic Genet.* 39, 194–199.
- Olivares-Gonzalez, L., de la Martinez-Fernandez, C., Hervas, D., Marin, M.P., Lahoz, A., Millan, J.M., Rodrigo, R., 2016. cGMP-phosphodiesterase inhibition prevents hypoxia-induced cell death activation in porcine retinal explants. *PLoS One* 11, e0166717.
- Orzalesi, N., Rossetti, L., Omboni, S., Group OS and Conproso, 2007. Vascular risk factors in glaucoma: the results of a national survey. *Graefes Arch. Clin. Exp. Ophthalmol.* 245, 795–802.
- Ozdegirmenci, O., Kucukozkan, T., Akdag, E., Topal, T., Haberal, A., Kayir, H., Oter, S., Akyol, M., Uzbay, T., 2011. Effects of sildenafil and tadalafil on ischemia/reperfusion injury in fetal rat brain. *J. Matern. Fetal Neonatal Med.* 24, 317–323.
- Ozel, A.B., Moroi, S.E., Reed, D.M., Nika, M., Schmidt, C.M., Akbari, S., Scott, K., Rozsa, F., Pawar, H., Musch, D.C., Lichter, P.R., Gaasterland, D., Branham, K., Gilbert, J., Garnai, S.J., Chen, W., Othman, M., Heckenlively, J., Swaroop, A., Abecasis, G., Friedman, D.S., Zack, D., Ashley-Koch, A., Ulmer, M., Kang, J.H., Liu, Y., Yaspan, B.L., Haines, J., Allingham, R.R., Hauser, M.A., Pasquale, L., Wiggs, J., Richards, J.E., Li, J.Z., 2014. Genome-wide association study and meta-analysis of intraocular pressure. *Hum. Genet.* 133, 41–57.
- Pacher, P., Beckman, J.S., Liaudet, L., 2007. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* 87, 315–424.
- Quigley, H.A., Broman, A.T., 2006. The number of people with glaucoma worldwide in 2010 and 2020. *Br. J. Ophthalmol.* 90, 262–267.
- Quigley, H.A., Nickells, R.W., Kerrigan, L.A., Pease, M.E., Thibault, D.J., Zack, D.J., 1995. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis. *Invest. Ophthalmol. Vis. Sci.* 36, 774–786.
- Reichstein, D., Ren, L., Filippopoulos, T., Mittag, T., Danias, J., 2007. Apoptotic retinal ganglion cell death in the DBA/2 mouse model of glaucoma. *Exp. Eye Res.* 84, 13–21.
- Rong, S.S., Tang, F.Y., Chu, W.K., Ma, L., Yam, J.C., Tang, S.M., Li, J., Gu, H., Young, A.L., Tham, C.C., Pang, C.P., Chen, L.J., 2016. Genetic associations of primary angle-closure disease: A systematic review and meta-analysis. *Ophthalmology* 123, 1211–1221.
- Santaella, R.M., Fraunfelder, F.W., 2007. Ocular adverse effects associated with systemic medications: recognition and management. *Drugs* 67, 75–93.
- Sappington, R.M., Chan, M., Calkins, D.J., 2006. Interleukin-6 protects retinal ganglion cells from pressure-induced death. *Invest. Ophthalmol. Vis. Sci.* 47, 2932–2942.
- Sappington, R.M., Carlson, B.J., Crish, S.D., Calkins, D.J., 2010. The microbead occlusion model: a paradigm for induced ocular hypertension in rats and mice. *Invest. Ophthalmol. Vis. Sci.* 51, 207–216.
- Sappington, R.M., Sidorova, T., Ward, N.J., Chakravarthy, R., Ho, K.W., Calkins, D.J., 2015. Activation of transient receptor potential vanilloid-1 (TRPV1) influences how retinal ganglion cell neurons respond to pressure-related stress. *Channels (Austin)* 9, 102–113.
- Sarhan, N.R., Omar, N.M., 2018. An immunohistochemical and ultrastructural analysis of the retina in tadalafil (Cialis) treated rats. *Acta Histochem.* 120, 312–322.
- Schallner, N., Romao, C.C., Biermann, J., Lagreze, W.A., Otterbein, L.E., Buerkle, H., Loop, T., Goebel, U., 2013. Carbon monoxide abrogates ischemic insult to neuronal cells via the soluble guanylate cyclase-cGMP pathway. *PLoS One* 8, e60672.
- Serarslan, Y., Yonden, Z., Ozgiray, E., Oktar, S., Guven, E.O., Sogut, S., Yilmaz, N., Yurtseven, T., 2010. Protective effects of tadalafil on experimental spinal cord injury in rats. *J. Clin. Neurosci.* 17, 349–352.
- Shaikh, Y., Yu, F., Coleman, A.L., 2014. Burden of undetected and untreated glaucoma in the United States. *Am J. Ophthalmol.* 158, 1121–1129 e1.
- Shoge, K., Mishima, H.K., Mukai, S., Shinya, M., Ishihara, K., Kanno, M., Sasa, M., 1999.

- Rat retinal ganglion cells culture enriched with the magnetic cell sorter. *Neurosci. Lett.* 259, 111–114.
- Sobrevia, L., Ooi, L., Ryan, S., Steinert, J.R., 2016. Nitric oxide: a regulator of cellular function in health and disease. *Oxidative Med. Cell. Longev.* 2016, 9782346.
- Stockman, A., Sharpe, L.T., Tufail, A., Kell, P.D., Ripamonti, C., Jeffery, G., 2007. The effect of sildenafil citrate (Viagra) on visual sensitivity. *J. Vis.* 7, 4.
- Tham, Y.C., Li, X., Wong, T.Y., Quigley, H.A., Aung, T., Cheng, C.Y., 2014. Global prevalence of glaucoma and projections of glaucoma burden through 2040: a systematic review and meta-analysis. *Ophthalmology* 121, 2081–2090.
- Thippeswamy, T., McKay, J.S., Morris, R., Quinn, J., Wong, L.F., Murphy, D., 2005. Glial-mediated neuroprotection: evidence for the protective role of the NO-cGMP pathway via neuron-glia communication in the peripheral nervous system. *GLIA* 49, 197–210.
- Thorleifsson, G., Walters, G.B., Hewitt, A.W., Masson, G., Helgason, A., DeWan, A., Sigurdsson, A., Jonasdottir, A., Gudjonsson, S.A., Magnusson, K.P., Stefansson, H., Lam, D.S., Tam, P.O., Gudmundsdottir, G.J., Southgate, L., Burdon, K.P., Gottfredsdottir, M.S., Aldred, M.A., Mitchell, P., St Clair, D., Collier, D.A., Tang, N., Sveinsson, O., Macgregor, S., Martin, N.G., Cree, A.J., Gibson, J., Macleod, A., Jacob, A., Ennis, S., Young, T.L., Chan, J.C., Karwatowski, W.S., Hammond, C.J., Thordarson, K., Zhang, M., Wadelius, C., Lotery, A.J., Trembath, R.C., Pang, C.P., Hoh, J., Craig, J.E., Kong, A., Mackey, D.A., Jonasson, F., Thorsteinsdottir, U., Stefansson, K., 2010. Common variants near CAV1 and CAV2 are associated with primary open-angle glaucoma. *Nat. Genet.* 42, 906–909.
- Tunny, T.J., Richardson, K.A., Clark, C.V., 1998. Association study of the 5' flanking regions of endothelial-nitric oxide synthase and endothelin-1 genes in familial primary open-angle glaucoma. *Clin. Exp. Pharmacol. Physiol.* 25, 26–29.
- ViagraR, 2008. Full Prescribing Information Sildenafil Citrate. Pfizer Inc., New York.
- Wareham, L.K., Buys, E.S., Sappington, R.M., 2018. The nitric oxide-guanylate cyclase pathway and glaucoma. *Nitric Oxide* 77, 75–87.
- Weinreb, R.N., Ong, T., Scassellati Sforzolini, B., Vittitow, J.L., Singh, K., Kaufman, P.L., 2015. group Vs. A randomised, controlled comparison of latanoprostene bunod and latanoprost 0.005% in the treatment of ocular hypertension and open angle glaucoma: the VOYAGER study. *Br. J. Ophthalmol.* 99, 738–745.
- Weinreb, R.N., Scassellati Sforzolini, B., Vittitow, J., Liebmann, J., 2016. Latanoprostene bunod 0.024% versus timolol maleate 0.5% in subjects with open-angle glaucoma and ocular hypertension: the APOLLO study. *Ophthalmology* 123, 965–973.
- Wiggs, J.L., Kang, J.H., Yaspan, B.L., Mirel, D.B., Laurie, C., Crenshaw, A., Brodeur, W., Gogarten, S., Olson, L.M., Abdrabou, W., DelBono, E., Loomis, S., Haines, J.L., Pasquale, L.R., 2011. Common variants near CAV1 and CAV2 are associated with primary open-angle glaucoma in Caucasians from the USA. *Hum. Mol. Genet.* 20, 4707–4713.
- Yucel, Y.H., Zhang, Q., Weinreb, R.N., Kaufman, P.L., Gupta, N., 2003. Effects of retinal ganglion cell loss on magno-, parvo-, koniocellular pathways in the lateral geniculate nucleus and visual cortex in glaucoma. *Prog. Retin. Eye Res.* 22, 465–481.
- Zarei, K., Scheetz, T.E., Christopher, M., Miller, K., Hedberg-Buenz, A., Tandon, A., Anderson, M.G., Fingert, J.H., Abramoff, M.D., 2016. Automated axon counting in rodent optic nerve sections with axon. *J. Sci. Rep.* 6, 26559.
- Zhao, D., Cho, J., Kim, M.H., Guallar, E., 2014. The association of blood pressure and primary open-angle glaucoma: a meta-analysis. *Am J. Ophthalmol.* 158, 615–627 e9.